## TITLE OF INVENTION

# IMMUNOGENIC COMPOSITIONS FOR PROTECTION AGAINST CHLAMYDIAL INFECTION

## FIELD OF THE INVENTION

The present invention relates to immunogenic compositions for protection against disease caused by *Chlamydia* infection in mammals, including humans.

## BACKGROUND OF THE INVENTION

Chlamydiae are procaryotes. They exhibit morphologic and structural similarities to gram-negative bacteria, including a trilaminar outer membrane, which contains lipopolysaccharide and several membrane proteins Chlamydiae are differentiated from other bacteria by their morphology and by a unique developmental cycle. They are obligate intracellular parasites with a unique biphasic life cycle consisting of a metabolically inactive but infectious extracellular stage and a replicating but non-infectious intracellular stage. The replicative stage of the life-cycle takes place within a membrane-bound inclusion which sequesters the bacteria away from the cytoplasm of the infected host cell.

Because chlamydiae are small and multiply only within susceptible cells, they were long thought to be viruses. However, they have many characteristics in common with other bacteria: (1) they contain both DNA and RNA, (2) they divide by binary fission, (3) their cell envelopes resemble those of other gram-negative bacteria, (4) they contain ribosomes similar to those of other bacteria, and (5) they are susceptible to various antibiotics. Chlamydiae can be seen in the light microscope, and the genome is about one-third the size of the *Escherichia coli* genome.

Many different strains of chlamydiae have been isolated from birds, man and other mammals, and these strains can be distinguished on the basis of host range, virulence, pathogenesis, and antigenic composition.

There is strong homology of DNA within each species, but surprisingly little between species, suggesting long-standing evolutionary separation.

*C. trachomatis* has a high degree of host specificity, being almost completely limited to man, and causes ocular and genitourinary infections of widely varying severity. In contrast, *C. psittaci* strains are rare in man but are found in a wide range of birds and also in wild, domestic, and laboratory mammals, where they multiply in cells of many organs.

*C. pneumoniae* is a common human pathogen, originally described as the TWAR strain of *C. psittaci*, but subsequently recognized to be a new species. *C. pneumoniae* is antigenically, genetically, and morphologically distinct from other *Chlamydia* species (*C. trachomatis, C. pecorum* and *C. psittaci*). It shows 10% or less DNA sequence homology with either of *C. trachomatis* or *C. psittaci* and so far appears to consist of only a single strain, TWAR.

*C. pneumoniae* is a common cause of community acquired pneumonia, less frequent only than *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* (refs. 1 and 2 - Throughout this application, various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosure of these references are hereby incorporated by reference into the present disclosure). *C. pneumoniae* can also cause upper respiratory tract symptoms and disease, including bronchitis and sinusitis (refs. 1 to 4). The great majority of the adult population (over 60%) has antibodies to *C. pneumoniae* (ref. 5), indicating past infection which was unrecognized or asymptomatic.

*C. pneumoniae* infection usually presents as an acute respiratory disease (*i.e.*, cough, sore throat, hoarseness, and fever; abnormal chest sounds on auscultation). For most patients, the cough persists for 2 to 6 weeks, and recovery is slow. In approximately 10% of these cases, upper respiratory tract infection is followed by bronchitis or pneumonia. Furthermore, during a *C. pneumoniae* epidemic, subsequent co-infection

with pneumococcus has been noted in about half of these pneumonia patients, particularly in the infirm and the elderly. As noted above, there is more and more evidence that *C. pneumoniae* infection is also linked to diseases other than respiratory infections.

The reservoir for the organism is presumably people. In contrast to *C. psittaci* infections, there is no known bird or animal reservoir. Transmission has not been clearly defined, but may result from direct contact with secretions, from formites, or from airborne spread. There is a long incubation period, which may last for many months. Based on analysis of epidemics, *C. pneumoniae* appears to spread slowly through a population (case-to-case interval averaging 30 days) because infected persons are inefficient transmitters of the organism. Susceptibility to *C. pneumoniae* is universal. Reinfections occur during adulthood, following the primary infection as a child. *C. pneumoniae* appears to be an endemic disease throughout the world, noteworthy for superimposed intervals of increased incidence (epidemics) that persist for 2 to 3 years. *C. trachomatis* infection does not confer cross-immunity to *C. pneumoniae*. Infections are easily treated with oral antibiotics, tetracycline or erythromycin (2 g/d, for at least 10 to 14 d). A recently developed drug, azithromycin, is highly effective as a single-dose therapy against chlamydial infections.

In most instances, *C. pneumoniae* infection is mild and without complications, and up to 90% of infections are subacute or unrecognized. Among children in industrialized countries, infections have been thought to be rare up to the age of 5 years, although a recent study has reported that many children in this age group show PCR evidence of infection despite being seronegative, and estimates a prevalence of 17 to 19% in 2 to 4 years old (ref. 6). In developing countries, the seroprevalence of *C. pneumoniae* antibodies among young children is elevated, and there are suspicions that *C. pneumoniae* may be an important cause of acute lower respiratory tract disease and mortality for infants and children in tropical regions of the world.

From seroprevalence studies and studies of local epidemics, the initial *C. pneumoniae* infection usually happens between the ages of 5 and 20 years. In the USA, for example, there are estimated to be 30,000 cases of childhood pneumonia each year caused by *C. pneumoniae*. Infections may cluster among groups of children or young adults (*e.g.*, school pupils or military conscripts).

*C. pneumoniae* causes 10 to 25% of community-acquired lower respiratory tract infections (as reported from Sweden, Italy, Finland, and the USA). During an epidemic, *C. pneumonia* infection may account for 50 to 60% of the cases of pneumonia. During these periods, also, more episodes of mixed infections with *S. pneumoniae* have been reported.

Reinfection during adulthood is common; the clinical presentation tends to be milder. Based on population seroprevalence studies, there tends to be increased exposure with age, which is particularly evident among men. Some investigators have speculated that a persistent, asymptomatic *C. pneumoniae* infection state is common.

In adults of middle age or older, *C. pneumoniae* infection may progress to chronic bronchitis and sinusitis. A study in the USA revealed that the incidence of pneumonia caused by *C. pneumoniae* in persons younger than 60 years is 1 case per 1,000 persons per year; but in the elderly, the disease incidence rose three-fold. *C. pneumoniae* infection rarely leads to hospitalization, except in patients with an underlying illness.

Of considerable importance is the association of atherosclerosis and *C. pneumoniae* infection. There are several epidemiological studies showing a correlation of previous infections with *C. pneumoniae* and heart attacks, coronary artery and carotid artery disease (refs. 7 to 11). Moreover, the organisms has been detected in atheromas and fatty streaks of the coronary, carotid, peripheral arteries and aorta (refs. 12 to 16). Viable *C. pneumoniae* has been recovered from the coronary and carotid artery. (refs, 17, 18). Furthermore, it has been shown that *C. pneumoniae* can induce changes of atherosclerosis in a rabbit model (ref. 19). Taken together, these results indicate that it is highly probable

that *C. pneumoniae* can cause atherosclerosis in humans, though the epidemiological importance of chlamydial atherosclerosis remains to be demonstrated.

A number of recent studies have also indicated an association between *C. pneumoniae* infection and asthma. Infection has been linked to wheezing, asthmatic bronchitis, adult-onset asthma and acute exacerbation of asthma in adults, and small-scale studies have shown that prolonged antibiotic treatment was effective at greatly reducing the severity of the disease in some individuals (refs. 20 to 25).

In light of these results, a protective vaccine against disease caused by *C. pneumoniae* infection would be of considerable importance. There is not yet an effective vaccine for human *C. pneumoniae* infection. Nevertheless, studies with *C. trachomatis* and *C. psittaci* indicate that this is an attainable goal. For example, mice which have recovered from a lung infection with *C. trachomatis* are protected from infertility induced by a subsequent vaginal challenge (ref. 26). Similarly, sheep immunized with inactivated *C. psittaci* were protected from subsequent chlamydial-induced abortions and stillbirths (ref. 27). Protection from chlamydial infections has been associated with Th1 immune responses, particularly the induction of INFγ-producing CD4+ T cells (ref. 28). The adoptive transfer of CD4+ cell lines or clones to nude or SCID mice conferred protection from challenge or cleared chronic disease (refs. 29, 30) and *in vivo* depletion of CD4+ T cells exacerbated disease post-challenge (refs. 31, 32). However, the presence of sufficiently high titres of neutralizing antibody at mucosal surfaces can also exert a protective effect (ref. 33).

The extent of antigenic variation within the species *C. pneumoniae* is not well characterized. Serovars of *C. trachomatis* are defined on the basis of antigenic variation in major outer membrane proteins (MOMP), but published *C. pneumoniae* MOMP gene sequences show no variation between several diverse isolates of the organism (refs. 34, 35, 36). Regions of the protein known to be conserved in other chlamydial MOMPs are conserved in *C. pneumoniae* (refs. 34, 35). One study has described a

strain of *C. pneumoniae* with a MOMP of greater that usual molecular weight, but the gene for this has not been sequenced (ref. 1). Partial sequences of outer membrane protein 2 from nine diverse isolates were also found to be invariant (ref. 17). The genes for HSP60 and HSP70 show little variation from other chlamydial species, as would be expected. The gene encoding a 76 kDa antigen has been cloned from a single strain of *C. pneumoniae*. It has no significant similarity with other known chlamydial genes (ref. 4).

Many antigens recognized by immune sera to *C. pneumoniae* are conserved across all chlamydiae, but 98kDa, 76 kDa and 54 kDa proteins may be *C. pneumoniae*-specific (refs. 2, 4, 37). Immunoblotting of isolates with sera from patients does show variation of blotting patterns between isolates, indicating that serotypes *C. pneumoniae* may exist (refs. 1, 17). However, the results are potentially confounded by the infection status of the patients, since immunoblot profiles of a patient's sera change with time post-infection. An assessment of the number and relative frequency of any serotypes, and the defining antigens, is not yet possible.

Thus, a need remains for effective compositions for preventing and treating *Chlamydia* infections.

## SUMMARY OF THE INVENTION

The present invention provides a novel approach to immunizing against Chlamydial infection based on nucleic acid immunization. It has surprisingly been found that the administration of a combination of nucleotide sequences encoding two different chlamydial proteins provides an enhanced protection efficacy.

Accordingly, in one aspect of the present invention, there is provided an immunogenic composition for *in vivo* administration to a host for the generation in the host of a protective immune response against Chlamydial infection, comprising a first vector comprising a first nucleotide sequence encoding a major outer membrane protein (MOMP) of a strain of *Chlamydia* and a first promoter sequence operatively coupled to said first nucleotide sequence for expression of said MOMP in the host; a second

vector comprising a second nucleotide sequence encoding a 76 kDa protein of a strain of *Chlamydia* and a second promoter sequence operatively coupled to said second nucleotide sequence for expression of said 76 kDa protein in the host; and a pharmaceutically-acceptable carrier therefor.

The first nucleotide sequence may encode a MOMP from any strain of *Chlamydia*, preferably from *C. pneumoniae* but also including *C. trachomatis*. The second nucleotide sequence encoding the MOMP protein of *C. pneumoniae* may have SEQ ID No: 12, 13 or 14 or may encode a MOMP having a SEQ ID No: 15 or 16.

The first promoter which is employed may be a cytomegalovirus promoter, although any other convenient promoter may be employed.

The second nucleotide sequence may encode a 76 kDa protein from any strain of *Chlamydia*, preferably from *C. pneumoniae* but also including *C. trachomatis*. The second nucleotide sequence encoding the 76 kDa protein of *C. pneumoniae* may have SEQ ID No: 1, 2, 3 or 4. The second nucleotide sequence may encode a 76 kDa protein having a molecular size of about 35 kDa and having SEQ ID No: 7 or may encode a 76 kDa protein having a molecular size of about 60 kDa and having SEQ ID No: 8 or 9.

The second promoter which is employed may be a cytomegalovirus promoter, although any other convenient promoter may be employed.

The first vector preferably comprises a plasmid vector and specifically may be pCAMOMP. Similarly the second vector preferably comprises a plasmid vector and specifically may be pCA76kDa. Most preferably, both the first and second vectors are plasmid vectors and specifically the combination of pCAMOMP and pCA76kDa.

The two vectors are used in an immunogenic composition along with any convenient pharmaceutically-acceptable carrier. As noted above, the uses of the combination of two vectors produces an enhanced protection efficacy in comparison to the individual vectors alone. Accordingly, the first and second vectors preferably are present in the

immunogenic composition in amounts such that the individual protective effect of each vector upon administration to the composition to the host is not adversely affected by the other.

The present invention, in a further aspect thereof, provides a method of immunizing a host against disease caused by infection with a strain of *Chlamydia*, which comprises administering to the host, which may be a human host, an effective amount of an immunogenic composition provided herein. The immunogenic composition preferably is administered intranasally.

## BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1 shows the nucleotide sequence of *C. pneumoniae* 76kDa gene (SEQ ID No: 1 - complete sequence; SEQ ID No: 2 - 5' encoding region; SEQ ID No: 3 - 3' encoding region including Myc and His encoding regions; SEQ ID No: 4 - 3' encoding region excluding Myc and His encoding regions; SEQ ID No: 5 - Myc encoding region; SEQ ID No: 6 - His encoding region) and the deduced amino acid sequences of two open reading frames of the 76kDa protein (SEQ ID NO: 7 - upstream reading frame; SEQ ID No: 8 - downstream reading frame including Myc and His regions; SEQ ID No: 9 - downstream reading frame excluding Myc and His regions; SEQ ID No: 10 - Myc region; SEQ ID No: 11 - His region);

Figure 2 shows a scheme of construction of plasmid pCA76kDa;

Figure 3 shows the nucleotide sequence of the *C. pneumoniae* MOMP gene (SEQ ID No: 12 - complete sequence; SEQ ID No: 13 - encoding sequence including Myc and His encoding regions; SEQ ID No: 14 - encoding sequence excluding Myc and His encoding regions) and the deduced amino acid sequence of the MOMP protein (SEQ ID No: 15 - including Myc and His regions; SEQ ID No: 16 - excluding Myc and His regions);

Figure 4 shows a scheme of the construction of plasmid pCAMOMP; and

Figure 5 illustrates the protective efficacy against *C. pneumoniae* lung challenge in Balb/c mice following DNA immunization with pCAMOMP plus pCA76kDa, in comparison to controls, wherein the individual data points (open diamonds) are shown for each animal, as well as the mean (solid squares) and standard deviation for each group.

## GENERAL DESCRIPTION OF INVENTION

As noted above, the present invention is directed to protecting a host against chlamydial infection by administering to the host an immunogenic composition containing two vectors, preferably plasmid vectors, each of which contains nucleotide sequence encoding a different protein of a strain of *Chlamydia*.

To illustrate the invention, a first plasmid vector was constructed containing the MOMP gene from *C. pneumoniae* and a second plasmid vector was constructed containing the 76 kDa protein gene from *C. pneumoniae*. While the invention is illustrated by the use of such plasmid vectors, other vectors containing such genes may be employed for administration to the host for expression of the encoded proteins in the host. Such other vectors may include live viral vectors, such as adenoviruses, alphaviruses including Semliki Forest virus and poxviruses including avipox and canary pox viruses as well as bacterial vectors, such as *Shigella*, *Salmonella*, *Vibrio cholerae*, *Lactobacillus*, Bacille Bilié de Calmette-Guérin (BCG) and *Streptococcus*.

One of the vectors employed herein contains a nucleic acid molecule which codes for a Chlamydial protein known in the art as the "76 kDa protein" (ref. 4). The latter terminology is utilized herein to refer to the protein identified in the art. Research has determined that the encoding nucleotide sequence for this protein in fact encodes two opening reading frames, one encoding a protein of approximately 35 kDa in length (SEQ ID No: 7) and the other encoding a protein of approximately 60 kDa in length (SEQ ID No: 9).

It has been found that, if the complete nucleotide sequence (SEQ ID No: 1) is incorporated into a suitable expression vector, then only the 35

kDa protein is expressed. If, however, the nucleotide sequence encoding the 60 kDa protein alone (SEQ ID No: 4) is incorporated into a suitable expression vector, then that protein also can be expressed. Both proteins have been found to be immunogenic and protective with the 35 kDa protein exhibiting a stronger protective effect than the 60 kDa protein (United States Patent Application No. 60/132,270 filed May 3, 1999; United States Patent Application No. 60/141,276 filed June 30, 1999, assigned to the Assignee hereof and the disclosures of which are incorporated herein by reference).

Any convenient plasmid vector may be used for the MOMP gene and the 76 kDa protein gene, such as the pcDNA3.1 expression vector (Invitrogen, San Diego, CA, USA) containing the cytomegalovirus promoter. Schemes for construction of the pCA76kDa plasmid vector of 8594 bp size and of the pCAMOMP plasmid vector of 7.6 kb in size, which include downstream DNA sequences coding for Myc and His tags, are shown in Figures 2 and 4 respectively and described in detail below.

The respective plasmids are formulated into an immunogenic composition in conjunction with a suitable pharmaceutically-acceptable carrier for administration to a host, such as a human host. The immunogenic composition may be administered in any convenient manner to the host, such as intramuscularly or intranasally, although other routes of administration may be used, as discussed below. The data presented herein and described in detail below demonstrates that DNA immunization with both the *C. pneumoniae* MOMP and 76 kDa protein genes elicits a strong protective immune response. The effect which is obtained is achieved without the use of adjuvant or other stimulation of immune response, such as cardiotoxin, although such materials may be used, if desired, as discussed below. In addition, the use of immunomodulation is not excluded from the scope of the invention. For example, it may be desirable to coadminister DNA that expresses immunoregulator cytokines (ref. 38).

As may be seen from the data below, by utilizing both the MOMP gene and the 76 kDa protein gene, there is obtained a protective immune response which is significantly greater than that achieved using the individual genes alone. The coadministration of the two genes does not result in any interference to the immune response of the individual genes.

There has previously been described in WO 98/02546, assigned to University of Manitoba and the disclosure of which is incorporated herein by reference, the use of the MOMP gene for DNA immunization. The improved results obtained herein using a combination of the MOMP gene and the 76 kDa protein gene demonstrate the use of multiple antigen genes from chlamydiae to augment the level of protective immunity achieved by DNA immunization. These results are more encouraging than those obtained using recombinant MOMP protein or synthetic peptides as the immunogen.

Nucleotide sequences, *e.g.*, DNA molecules, can easily be retrieved by polymerase chain reaction (PCR) amplification of genomic bacterial DNA extracted by conventional methods. This involves the use of synthetic oligonucleotide primers matching upstream and downstream of the 5' and 3' ends of the encoding domain. Suitable primers can be designed according to the nucleotide sequence information provided. Typically, a primer can consist of 10 to 40, preferably 15 to 25 nucleotides. It may be also advantageous to select primers containing C and G nucleotides in a proportion sufficient to ensure efficient hybridization; *e.g.*, an amount of C and G nucleotides of at least 40%, preferably 50% of the total nucleotide amount.

It is clearly apparent to one skilled in the art that the various embodiments of the present invention have many applications in the fields of vaccination and treatment of chlamydial infection. A further non-limiting discussion of such uses is further presented below.

**1. Vaccine Preparation and Use**

Immunogenic compositions, suitable to be used as vaccines, may be prepared from the MOMP gene and the 76 kDa protein gene and vectors as

disclosed herein. The vaccine elicits an immune response in a subject which includes the production of anti-MOMP and anti-76 kDa protein antibodies. Immunogenic compositions, including vaccines, containing the nucleic acid may be prepared as injectables, in physiologically-acceptable liquid solutions or emulsions for polynucleotide administration.

The nucleic acid may be associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a nucleic acid liposome (for example, as described in WO 93/24640) or the nucleic acid may be associated with an adjuvant, as described in more detail below. Liposomes comprising cationic lipids interact spontaneously and rapidly with polyanions, such as DNA and RNA, resulting in liposome/nucleic acid complexes that capture up to 100% of the polynucleotide. In addition, the polycationic complexes fuse with cell membranes, resulting in an intracellular delivery of polynucleotide that bypasses the degradative enzymes of the lysosomal compartment.

Published PCT application WO 94/27435 describes compositions for genetic immunization comprising cationic lipids and polynucleotides. Agents which assist in the cellular uptake of nucleic acid, such as calcium ions, viral proteins and other transfection facilitating agents, may advantageously be used.

Polynucleotide immunogenic preparations may also be formulated as microcapsules, including biodegradable time-release particles. Thus, U.S. Patent 5,151,264 describes a particulate carrier of a phospholipid/glycolipid/polysaccharide nature that has been termed Bio Vecteurs Supra Moléculaires (BVSM). The particulate carriers are intended to transport a variety of molecules having biological activity in one of the layers thereof.

U.S. Patent 5,075,109 describes encapsulation of the antigens trinitrophenylated keyhole limpet hemocyanin and staphylococcal enterotoxin B in 50:50 poly (DL-lactideco-glycolide). Other polymers for encapsulation are suggested, such as poly(glycolide), poly(DL-lactide-co-glycolide), copolyoxalates, polycaprolactone, poly(lactide-co-caprolactone),

poly(esteramides), polyorthoesters and poly(8-hydroxybutyric acid), and polyanhydrides.

Published PCT application WO 91/06282 describes a delivery vehicle comprising a plurality of bioadhesive microspheres and antigens. The microspheres being of starch, gelatin, dextran, collagen or albumin. This delivery vehicle is particularly intended for the uptake of vaccine across the nasal mucosa. The delivery vehicle may additionally contain an absorption enhancer.

The vectors may be mixed with pharmaceutically acceptable excipients which are compatible therewith. Such excipients may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously, intravenously, intradermally, intraperitoneally or intramuscularly, possibly following pretreatment of the injection site with a local anesthetic.

Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the ocular, pulminary, nasal or oral (intragastric) routes. Alternatively, other modes of administration including rectal, vaginal or urinary tract as well as suppositories may be desirable. For suppositories, binders and carriers may include, for example, polyalkylene glycols or triglycerides. Oral formulations may include normally employed incipients, such as, for example, pharmaceutical grades of saccharine, cellulose and magnesium carbonate.

The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as is therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize the MOMP and

76 kDa proteins and antibodies thereto, and if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgement of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of about 1 μg to about 1 mg of the vectors.

Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host. A vaccine which protects against only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and also belong to the present invention. Such combined vaccines contain, for example, material from various pathogens or from various strains of the same pathogen, or from combinations of various pathogens.

Immunogenicity may be significantly improved if the vectors are co-administered with adjuvants, commonly used as 0.05 to 0.1 percent solution in phosphate-buffered saline. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Thus, adjuvants have been identified that enhance the immune response to antigens. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines.

A wide range of extrinsic adjuvants and other immunomodulating material can provoke potent immune responses to antigens. These include

saponins complexed to membrane protein antigens to produce immune stimulating complexes (ISCOMS), pluronic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as Quil A derivatives and components thereof, QS 21, calcium phosphate, calcium hydroxide, zinc hydroxide, an octodecyl ester of an amino acid, ISCOPREP, DC-chol, DDBA and polyphosphazene. Advantageous combinations of adjuvants are described in copending United States Patent Applications Nos.: 08/261,194 filed June 16, 1994 and 08/483,856 filed June 7, 1995, assigned to the assignee hereof and the disclosures of which are incorporated herein by reference thereto (WO 95/34308).

In particular embodiments of the present invention, the vectors may be delivered in conjunction with a targeting molecule to target the vectors to selected cells including cells of the immune system.

The vectors may be delivered to the host by a variety of procedures, for example, Tang et al. (ref. 39) disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH antibodies in the mice, while Furth et al. (ref. 40) showed that a jet injector could be used to transfect skin, muscle, fat and mammary tissues of living animals. See also U.S. Patents Nos. 4,245,050 and 5,015,580 and WO 94/24263.

## EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples. These examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

Example 1:

This Example illustrates the preparation of a plasmid vector pCA76kDa containing the 76kDa protein gene.

The 76kDa protein gene was amplified from *Chlamydia pneumoniae* (CM1) genomic DNA by polymerase chain reaction (PCR) using a 5' primer (5' GCTCTAGA*CCGCC***ATGACAAAAAAACAT TATGCTTGGG** 3') (SEQ ID No: 9) and 3' primer (5' CGGGATCCA**TAGAACTTGCTGCAGCGGG** 3') (SEQ ID No: 10). The 5' primer contains a Xba I restriction site, a ribsome binding site, an initiation codon and a sequence close to the 5' end of the 76kDa protein coding sequence. The 3' primer includes the sequence encoding the C-terminal sequence of the 76kDa protein and a Bam HI restriction site. The stop codon was excluded and an additional nucleotide was inserted to obtain an inframe C-terminal fusion with the Histidine tag. The presence of a stop codon at nucleotide 828 of the amplified sequence means that only a partial 76kDa protein is expressed.

After amplification, the PCR fragment was using QIAquick™ PRC purification kit (Qiagen) and then digested with Xba I and Bam HI and cloned into the pCA-Myc-His eukaryotic expression vector as described in Example 3 below (Figure 2) with transcription under control of the human CMV promoter.

Example 2:

This Example illustrates the preparation of a plasmid vector pCAMOMP containing the MOMP protein gene.

The MOMP protein gene was amplified from *Chlamydia pneumoniae* (CM1) genomic DNA by polymerase chain reaction (PCR) using a 5' primer (5' CCCGGATATC*CCACC***ATGTTGCCTGTAGG GAACCCTTC** 3') (SEQ ID No: 11) and a 3' primer (5' GGGGTACCG**GAATCTGAACTGACCAGATACG** 3') (SEQ ID No: 12). The 5' primer contains a EcoRV restriction site, a ribosome binding site, an initiation codon and a sequence encoding the N-terminal sequence of the mature MOMP. The 3' primer includes the sequence encoding the C-

terminal sequence of the MOMP and a Kpn I restriction site. The DNA sequence encoding the leader peptide was excluded, the stop codon was excluded and an additional nucleotide was inserted to obtain an in-frame C-terminal fusion with the Histdine tag.

After amplification, the PCR fragment was purified using QIAquick™ PCR purification kit (Qiagen) and then digested with Eco RV and Kpn I and cloned into the pCA-Myc-His eukaryotic expression vector described in Example 3 (Figure 4) with transcription under control of the human CMV promoter.

Example 3:

This Example illustrates the preparation of the eukaryotic expression vectors pCA76kDa and pCAMOMP.

Plasmid pcDNA3.1 (–) (Invitrogen) was restricted with Spe I and Bam HI to remove the CMV promoter and the remaining vector fragment was isolated. The CMV promoter and intron A from plasmid VR-1012 (Vical) was isolated on a Spe I / Bam HI fragment. The fragments were ligated together to produce plasmid pCA/Myc-His, as seen in Figure 2.

The Xba I/Bam HI restricted PCR fragment containing the 76kDa protein gene (Example 1) was ligated into the Xba I and Bam HI restricted plasmid pCA/Myc-His to produce plasmid pCA76kDa (Figure 2).

The Eco RV/Kpn I restricted PCR fragment containing the MOMP gene (Example 2) was ligated into Eco RV/Kpn I restricted pCA/Myc-His to produce plasmid pCAMOMP (Figure 4).

The resulting plasmids, pCA76kDa and pCAMOMP, were transferred by electroporation into *E. coli* XL-1 blue (Stratagene) which was grown in LB broth containing 50 μg/ml of carbenicillin. The plasmids were isolated by Endo Free Plasmid Giga Kit™ (Qiagen) large scale DNA purification system. DNA concentration was determined by absorbance at 260 nm and the plasmid was verified after gel electrophoresis and Ethidium bromide staining and comparison to molecular weight standards. The 5' and 3' ends of the gene were verified by sequencing using a LiCor model 4000 L DNA sequencer and IRD-800 labelled primers.

Example 4:

This Example illustrates the immunization of mice to achieve protection against an intranasal challenge by *C. pneumoniae*.

It has been previously demonstrated that mice are susceptible to intranasal infection with different isolates of *C. pneumoniae* (ref. 41). Strain AR-39 (ref. 42) was used in Balb/c mice as a challenge infection model to examine the capacity of chlamydia gene products delivered as naked DNA to elicit a protective response against a sublethal *C. pneumoniae* lung infection. Protective immunity is defined as an accelerated clearance of pulmonary infection.

Groups of 7 to 9 week old male Balb/c mice (5 to 9 per group) were immunized intramuscularly (i.m.) and intranasally (i.n.) with plasmids pCA76kDa and pCAMOMP containing the coding sequences of *C. pneumoniae* 76kDa and MOMP, respectively, prepared as described in Example 3. Saline or plasmid vectors containing non-protective inserted chlamydial genes, namely pCAI116 and pCAI178,were given to groups of control animals.

The constructs pCAI116 and pCAI178 are identical to pCA76kDa and pCAMOMP except that the nucleotide sequence encoding the partial 76kDa protein or MOMP is replaced with a *C. pneumoniae* nucleotide sequence encoding, respectively, a possible inclusion membrane protein and a nucleoside 5'-diphosphate phosphotransferase, respectively.

For i.m. immunization, alternate left and right quadriceps were injected with 100 μg of each DNA construct in 50 μl of PBS on three occasions at 0, 3, and 6 weeks. For i.n. immunization, anaesthetized mice aspirated 50 μl of PBS containing 50 μg of each DNA construct on three occasions at 0, 3, and 6 weeks. At week 8, immunized mice were inoculated i.n. with $5 \times 10^5$ IFU of *C. pneumoniae*, strain AR39, in 100 μl of SPG buffer to test their ability to limit the growth of a sublethal *C. pneumoniae* challenge.

Lungs were taken from mice at day 9 post-challenge and immediately homogenized in SPG buffer (7.5% sucrose, 5 mM glutamate,

12.5 mM phosphate, pH 7.5). The homogenate was stored frozen at –70°C until assay. Dilutions of the homogenate were assayed for the presence of infectious chlamydia by inoculation onto monolayers of susceptible cells. The inoculum was centrifuged onto the cells at 3000 rpm for 1 hour, then the cells were incubated for three days at 35°C in the presence of 1 μg/ml cycloheximide. After incubation, the monolayers were fixed with formalin and methanol, then immunoperoxidase stained for the presence of *Chlamydial* inclusions using convalescent sera from rabbits infected with *C. pneumoniae* and metal-enhanced DAB as a peroxidase substrate.

Figure 5 and Table 1 contain the results obtained and show that mice immunized i.n. and i.m. with both pCA76kDa and pCAMOMP had chlamydial lung titers less than 6700 in 6 of 6 cases, whereas the range of values for control mice with saline were 15,000 to 106,100 IFU/lung in 20 out of 23 cases (mean 49,000) and 12,600 to 80,600 IFU/lung in 11 out of 12 cases (mean 33,500 to 47,000) for mice immunized with the vectors containing non-protective genes (Table 1). The mice immunized with only the pCAMOMP alone showed lung titres in the range of 5800 to 18,700 in 5 out of 6 cases (mean 12,600) and mice immunized with pCA76kDa alone showed similar titres in the range of 6,300 to 18,200 in 5 out of 6 cases (mean 7,400). The increased protection afforded by the combination of the two constructs is surprising in light of other failures due to antigen competition.

Table 1

| MOUSE | BACTERIAL LOAD (INCLUSION FORMING UNITS PER LUNG) IN THE LUNGS OF BALB/C MICE IMMUNIZED WITH VARIOUS DNA IMMUNIZATION CONSTRUCTS | | | | | |
|---|---|---|---|---|---|---|
| | IMMUNIZING CONSTRUCT | | | | | |
| | Saline | pCAI116 | pCAI178 | pCAMOMP | pCA76kDa | pCAMOMP + pCA76kDa |
| | **Day 9** | **Day 9** | **Day 9** | **Day 9** | **Day 9** | **Day 9** |
| 1 | 1700 | 47700 | 80600 | 5800 | 18200 | 6600 |
| 2 | 36200 | 12600 | 31900 | 30200 | 6300 | 5300 |
| 3 | 106100 | 28600 | 30600 | 9900 | 13400 | 0 |
| 4 | 33500 | 17700 | 6500 | 18700 | 100 | 3300 |
| 5 | 70400 | 77300 | 53000 | 0 | 2400 | 5200 |
| 6 | 48700 | 17600 | 79500 | 11000 | 4000 | 2700 |
| 7 | 600 | | | | | |
| 8 | 19800 | | | | | |
| 9 | 29500 | | | | | |
| 10 | 100000 | | | | | |
| 11 | 15000 | | | | | |
| 12 | 56600 | | | | | |
| 13 | 60300 | | | | | |
| 14 | 88800 | | | | | |
| 15 | 30400 | | | | | |
| 16 | 69300 | | | | | |
| 17 | 47500 | | | | | |
| 18 | 96500 | | | | | |
| 19 | 30200 | | | | | |
| 20 | 84800 | | | | | |
| 21 | 3800 | | | | | |
| 22 | 65900 | | | | | |
| 23 | 33000 | | | | | |
| | | | | | | |
| MEAN | 49069.57 | 33583.33 | 47016.67 | 12600 | 7400 | 3850 |
| SD | 32120.48 | 24832.67 | 29524.32 | 10600.19 | 6981.40 | 2363.68 |

## SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides a novel immunization procedure for obtaining an enhanced protective immune response to Chlamydial infection by employing DNA immunization using nucleotide sequences encoding a MOMP and a 76 kDa protein of a strain of *Chlamydia*. Modifications are possible within the scope of the invention.

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